A simple and rapid method for sequencing DNA

S.A. Chuvpilo and V.V. Kravchenko*

*M.M. Shemyakin Institute of Bioorganic Chemistry, Academy of Sciences of the USSR, Moscow, and *All-Union Research Institute of Molecular Biology, Koltsovo, Novosibirsk Region, USSR*

Received 22 October 1984

A simplified technique for DNA sequence analysis has been developed, based on modification of a previous method [(1980) Methods Enzymol. 65, 499–560]. It employs an adsorptive immobilization of terminally labelled DNA on DEAE paper followed by G, A+G, C+T and C specific modification and cleavage reactions. This solid-phase technique is faster and more convenient than the original method. The efficiency is comparable. The total processing time taken to produce cleaved fragments loaded on a gel is less than 2 h.

DNA sequencing *DNA adsorptive immobilization*

1. INTRODUCTION

At present, the most popular method for DNA sequence determination is probably that of [1]. It is very simple and efficient but involves numerous DNA precipitation and lyophilization steps. These are essential since incomplete removal of salts (before modification of DNA) or chemical reagents (after the modification and piperidine cleavage) impairs the sequencing gel and often produces artifactual bands.

Here, we describe a simplified solid-phase technique for DNA sequencing, which is as efficient as the original method [1] but is much faster and less laborious. This solid-phase approach employs an adsorptive immobilization of a terminally labelled DNA fragment on DEAE paper followed by specific chemical modification and cleavage reactions.

2. MATERIALS AND METHODS

^{32}P-labelling of DNA and gel electrophoresis were carried out as described [1]. RNA carrier was prepared by digestion of 10 mg yeast tRNA (Sigma) in 1 ml of water with 20 μg RNase A (Boehringer) for 30 min at 37°C to reduce the homogeneity of the nucleic acid; the protein was removed by phenol-chloroform (1:1, v/v) extraction and the hydrolysate precipitated twice from 0.3 M sodium acetate, pH 5.5. DEAE paper DE 81 was purchased from Whatman; ^{32}P-labelled nucleoside 5′-triphosphates were from Amersham International; dimethyl sulfate, formic acid, hydrazine hydrate, and piperidine were from BDH. All other reagents were from BRL.

2.1. *Sample preparation*

To adsorb on DEAE paper, a terminally ^{32}P-labelled DNA fragment was electroeluted from a polyacrylamide gel in an ISCO sample concentrating apparatus with a DEAE paper disc (~5 mm in diameter) placed between the apparatus outlet and membrane bag. The elution buffer was 25 mM Tris borate (pH 8.3) and 0.5 mM EDTA. The elution at 250 v was complete in 1–2 h for 100–1000 bp long fragments. If Bio-Rad or Reanal electrophoresis apparatus are employed instead of an ISCO concentrator, an ordinary glass tube closed by the DEAE paper disc and dialysis membrane can be used. After the elution was complete, the disc was rinsed in distilled water for 1–2 s and put on a blotting paper, the procedure being repeated 5 times. The the disc was washed in

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a similar manner with 96% ethanol. The discs were dried, cut into 4 pieces and each piece was subjected to a different chemical modification.

Alternatively, DNA fragments were eluted from the gel as described in [1], ethanol precipitated in the presence of 50 μg RNA carrier and dissolved in 20 μl water. Aliquots (5 μl) were applied to 4 marked DEAE paper strips (2.5 × 5 mm). Washing was performed as above.

2.2. *Chemical modification*

Four base-specific chemical modification reactions were utilised: Guanine-specific modification was achieved using 1% dimethyl sulfate in 50 mM ammonium formate buffer (pH 3.5) [2]; G+A specific modification (depurination) using 66% formic acid [3]; C+T specific modification using hydrazine hydrate [3]; and C-specific modification using hydrazine hydrate saturated with NaCl containing 0.25 M NaOH [3].

Reactions were performed on dry strips placed on Saran Wrap. Strips were soaked with a volume of reagent such that it was completely adsorbed by the paper.

Excess reagent should be avoided, especially for the C-specific reaction, since desorption of DNA can occur. Samples were covered with an additional piece of Saran Wrap to prevent drying.

After modification the strips were washed 5 times with ethanol, 70% aqueous ethanol, water and finally with ethanol. The order of solvents is essential to prevent losses. The strips were then dried.

Table 1

Optimal times for DNA modification reactions at room temperature

Distance from the labelled end (nucleotide number)	Time (min)			
	G	A+G	C+T	C
>250	1	2.5	5	5
120–250	2	5	10	10
70–120	4	10	20	20
30–70	6	15	30	30
15–30	8	20	40	40
10–15	12	30	60	60

Optimal times for the reactions held on solid phase are similar to those carried out in solution and are specified in table 1.

2.3. *Hydrolysis and desorption of DNA*

All paper strips were placed together into a 1.5 ml polypropylene Eppendorf test tube and heated with 1 M aqueous piperidine for 30 min at 100°C. Piperidine was removed by sequential washing with ethanol and water, then dried. Each strip was put into a yellow pipette tip (200 μl) inserted into a 0.4 ml polypropylene tube. Each sample was eluted for 10 min at 65°C with 50 μl 1 M NaCl solution containing 10 mM EDTA and 5 μg RNA carrier. Samples were centrifuged to transfer the eluate into the tube. The desorption procedure was repeated, then radioactive material was precipitated from the eluate using 96% ethanol. Samples were washed with 70% ethanol, dried, dissolved in formamide and loaded on a polyacrylamide gel. Electrophoresis was carried out as described [1].

3. RESULTS AND DISCUSSION

To simplify chemical sequencing DNA was immobilized by adsorption on DEAE paper. All the modification reactions, washing, etc., were performed on solid support rather than in solution. The advantage of this technique is that one can

Table 2

Radioactivity (cpm) adsorbed on DEAE paper during solid-phase sequencing

	Type of reaction			
	G	A+G	C+T	C
Before modification	56800	54300	55300	48700
After modification and cleavage by piperidine	52000	53900	53500	42500
Desorbed from the paper	44100	41000	40700	31400
Left on the paper	7800	11800	12600	11000

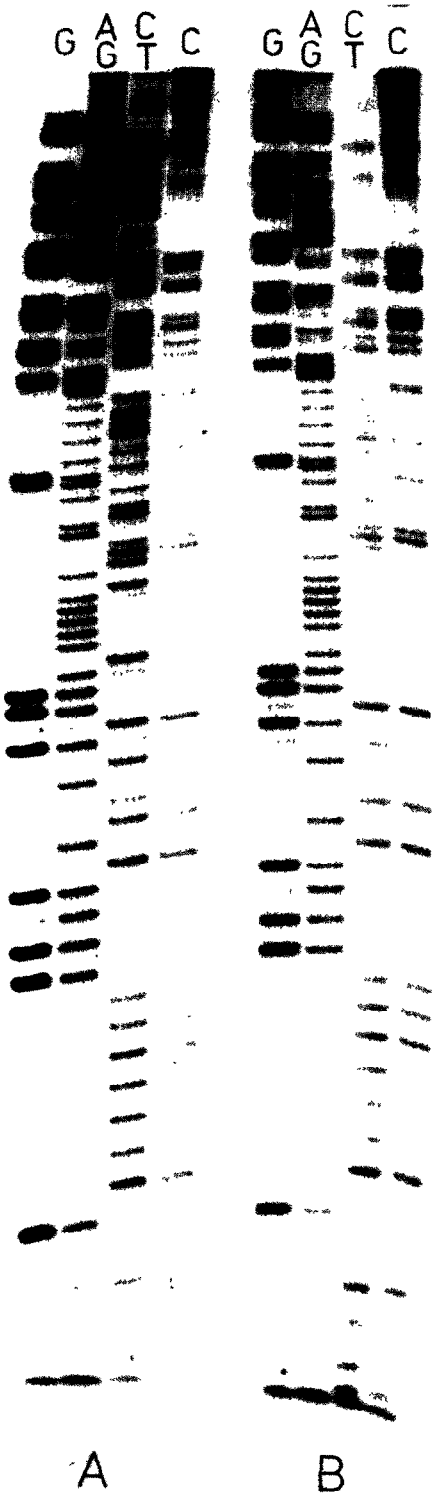

Fig.1. Autoradiograph of a gel on sequencing an EcoRI/MspI fragment of plasmid pBR322. A, sequencing on DEAE paper. B, sequencing in solution.

easily operate with the strips of paper, which makes the analysis much faster and less laborious. For example, the removal of piperidine by this technique is complete in 1–2 min whereas it takes several hours by the protocol of [1]. Repeated ethanol precipitations of DNA needed for removal of modifying reagents are substituted by brief washing of paper strips. Time for washing does not depend on the number of samples if they are treated together in an ultrafiltration vacuum-cell.

Approximately one quarter of the starting radioactivity is lost during the solid-phase sequencing (table 2). The main loss occurs due to incomplete desorption of DNA from DEAE paper rather than to the chemical reactions of modification and piperidine cleavage. The irreversible sorption of DNA can be reduced by prewashing DEAE paper with 1 M NaCl solution and then with water.

Comparison of the results obtained by our solid-phase technique and the method of [1] (see fig.1) shows that immobilization of DNA on DEAE paper does not affect the specificity of chemical modification and cleavage.

High resolution of the bands on the autoradiograph is indicative of the adequate removal of the impurities that might impair the sequencing gel.

The solid-phase technique is therefore a simple and time-saving alternative to the traditional solution method for sequencing DNA.

ACKNOWLEDGEMENTS

The authors are thankful to Drs V.G. Korobko and V.N. Dobrynin for helpful and critical discussions.

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